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Long term potentiation and synaptic protein phosphorylation

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Introduction

Biochemical processes underlying memory processes have been subject to study for many years. Protein synthesis and posttranslational modifications of proteins (like phosphorylation or glycosylation) have been found to change in correlation with learning and memory (Teyler and DiScenna, 1987; Schwartz and Greenberg, 1987). Simple behavioral modifications, such as sensitization and classical conditioning can be related to changes in the plasticity of previously formed synaptic connections. While protein synthesis seems to be required for long-term changes in neurotransmission lasting up to periods of days (Frey et al., 1988; Goelet et al., 1986), phosphorylation of proteins, thereby changing their activity, seems to be related to shorter term responses (Schwartz and Greenberg, 1987). Phosphorylation of ion channels has been shown to regulate their function, thereby changing neuroexcitability, action potential amplitude and duration, and neurotransmitter (NT) release (Levitan, 1985). Several protein kinases have been described to participate in potentiation of neurotransmission by changing ion channel function and/or facilitating release of NT, among which are cyclic-AMP dependent protein kinase (A-kinase), Ca^{2+}/calmodulin-dependent protein kinase (CaM KII) and protein kinase C (PKC) (Schwartz and Greenberg, 1987; Miller, 1986; Kaczmarek, 1987). In mammalian systems attention has recently focussed mainly on the role of PKC in long-term potentiation (LTP) of synaptic transmission.

PKC is a Ca^{2+}/phospholipid-dependent kinase, stimulated by diacylglycerol (DG). Activation of PKC is believed to involve either translocation of the kinase from the cytoplasm to the plasma membrane or tighter binding to the membrane, were it binds to phospholipids (preferably phosphatidylserine, Nishizuka, 1986). DG produced by phospholipase C-catalyzed hydrolysis of polyphosphoinositides in the membrane, activates the kinase by lowering the requirements for Ca^{2+} (Nishizuka, 1986). The kinase also can be activated by DG analogues such as 1,2-dioctanoylglycerol (DOG) and by tumor-promoting phorbol esters.

Activators of PKC (phorbol esters) give rise to electrophysiological changes similar to those observed after tetanization of hippocampal slice preparation (Malenka et al., 1986)

and inhibitors of PKC (polymyxin B, H-7, mellitin) attenuate LTP (Lovinger et al., 1987; Reymann et al., 1988a). Eyelid conditioning in the rabbit gives rise to an increase in PKC activity associated with the membrane in the CA1 region of the hippocampus, tested 24h after conditioning (Bank et al., 1988). Furthermore, injection of the purified kinase in hippocampal pyramidal cells elicits features of LTP (Hu et al., 1987).

The two major neuronal PKC substrates identified in the nervous system are an 87 kDa protein and a 48 kDa protein called B-50/F1/GAP-43. The phosphorylation of the 87 kDa protein is stimulated by phorbol esters and depolarization of synaptosomes (Wu et al., 1982). The second PKC substrate is a membrane-associated neuron-specific protein that is localized presynaptically (Gispen et al., 1985). This protein is identical to F1, GAP-43, GAP-48 and pp46 and will further be referred to as B-50.

Various studies have pointed to an important role for B-50 in different forms of plasticity of the nervous system (Benowitz and Routtenberg, 1987). Recently Routtenberg's group has shown that after eliciting LTP *in vivo* in the hippocampus PKC activity translocates from the cytoplasm to the particulate (membrane) fraction as measured *in vitro* (Akers et al., 1986). In this same *in vivo/in vitro* approach, an increased post-hoc phosphorylation of B-50 was observed (Routtenberg, 1985; Lovinger et al., 1986). The increase in *in vitro* phosphorylation was found to be persistent and directly related to the degree of potentiation. It is not known yet, whether the increase in *in vitro* phosphorylation of B-50 found after tetanization also occurs *in vivo*. The increase in B-50 phosphorylation measured in a broken cell preparation may merely reflect the increase in PKC activity at the membrane. To investigate whether an increase in B-50 phosphorylation may be part of the molecular mechanisms underlying LTP, we measured B-50 phosphorylation in intact hippocampal slices prelabelled with $^{32}P_i$ using a quantitative immunoprecipitation assay (De Graan et al., 1988a).

Earlier studies on the role of phosphorylation in LTP have revealed two other phosphoproteins to be correlated with LTP; the α-subunit of the multi-enzyme complex pyruvate dehydrogenase (α-PDH) and a 52 kDa coated vesicle protein. The proposed function of these phosphoproteins as well as their possible involvement in LTP will be discussed.

The data presented in this paper will be restricted to mammalian systems. More detailed information on the role on especially protein kinases but also their substrates has been obtained in invertebrate systems (e.g. Hermissenda, Aplysia etc.), where the function of individual nerve cells is better characterized and more insight has been gained on the regulation of ion channels by phosphorylation.

Results

Pyruvate dehydrogenase phosphorylation and LTP

In search of biochemical correlates of LTP, Browning et al. (1979) studied protein phosphorylation in crude synaptosomal/mitochondrial fractions prepared from hippocampal slices. High frequency stimulation (100 Hz for 1s) applied to the Schaffer collaterals decreased the amount of phosphate in a 40 kDa protein band compared to controls measured by a post-hoc phosphorylation assay. Omission of Ca^{2+} from the extracellular medium prohibited both the induction of LTP and the effect on the 40 kDa protein. The maximal effect on the 40 kDa band was observed only shortly after the tetanus and was abolished 15 min thereafter (Browning et al., 1982). The 40 kDa protein was found to be confined to the mitochondrial fraction upon subcellular fractionation (Browning et al., 1981a) and resembled characteristics of the α-subunit of pyruvate dehydrogenase (α-PDH), an enzyme involved in pyruvate decarboxylation and Ca^{2+} sequestra-

tion into mitochondria. The 40 kDa protein comigrated with α-PDH isolated from kidney as was shown for protein band F_2 (Morgan and Routtenberg, 1980). The activity of α-PDH kinase can be inhibited by dichloroacetate and pyruvate, both conditions leading to an activation of PDH activity and a decreased phosphorylation of the enzyme (Browning et al., 1981b), whereas inhibitors of α-PDH activity such as NADH and acetyl CoA stimulate α-PDH kinase (Morgan and Routtenberg, 1980).

Possible involvement of the major coated vesicle phosphoprotein in LTP

Tetanic stimulation of the perforant path of rat hippocampal slices (15 pulses/s, 15 s) increases the phosphorylation of a 50 kDa protein in a crude synaptosomal/mitochondrial preparation (Bär et al., 1980) prepared 15 min after tetanization, when assayed in the absence of Ca^{2+}. It was demonstrated that the increase in phosphorylation of this protein (24% compared to non-stimulated controls) was dependent on the frequency of the tetanus used, at 1 pulse/s for 15 s no effect of tetanus could be seen (Bär et al., 1980). Moreover, the increase in 50 kDa phosphorylation after tetanization was dependent on the presence of extracellular Ca^{2+}, indicating the necessity of LTP production and neurotransmission for the observed effect (Dunwiddie and Lynch, 1979). Using an improved separation system it could be shown that the 50 kDa phosphoprotein actually consisted of two bands of 52 and 50 kDa respectively and only the 52 kDa band was found to be susceptible to LTP. Subcellular fraction studies revealed this 52 kDa band to be present in a purified synaptosomal plasma membrane (SPM) fraction (Bär et al., 1982).

The electrophysiological changes to a tetanus as determined by measuring the evoked response to a single test stimulus may vary substantially. Therefore we performed a quantitative study on the change in the post-hoc phosphorylation of the 52 kDa protein and the changes in the amplitude of the population spike (PS) and the postsynaptic potential (PSP) (Tielen et al., 1983). Two high-frequency stimuli (50 pulses/s, 2s) were given 5 min apart. Ten min after the second stimulus, the slices were processed individually and assayed for endogenous protein phosphorylation. The unstimulated phosphate incorporation was determined in the paired contralateral slice after application of only test stimuli. The percentual change in the post-hoc phosphorylation of the 52 kDa band was correlated to the change in PS amplitude in each individual slice. A semi-logarithmic plot of the data fitted a straight line with a correlation coefficient of 0.71 ($p < 0.005$) suggesting that there may be a quantitative relationship between the change in electrophysiological characteristics and biochemical properties of the membrane 10 min after presenting the tetanus.

In a first attempt to elucidate the identity of the 52 kDa protein, we studied its phosphorylation characteristics. The *in vitro* phosphorylation of the 52 kDa protein was not dependent of Ca^{2+}/calmodulin or cyclic-AMP nor was it dependent upon Ca^{2+} tested in a range of 0–50 mM in the presence of 1 mM EGTA (Bär et al., 1982). At the same time the phosphorylation of another protein of 50 kDa was described (Pauloin et al., 1982). This protein was the major coated vesicle (CV) phosphoprotein and its *in vitro* phosphorylation was, similar to that of the 52 kDa protein, independent of Ca^{2+} and cyclic-AMP (Schrama et al., 1986a). Coated vesicles are a special class of intracellular organelles which have been implicated in a number of intracellular processes such as receptor-mediated endocytosis and presynaptic membrane recycling after NT release. In a biochemical study the characteristics of the 52 kDa phosphoprotein from SPM and phosphorylated pp50 from CVs were compared (Schrama et al., 1986b). The proteins appeared to be similar with respect to the following properties: molecular weight was 52 kDa on an 11% SDS-polyacrylamide gel, the phospho amino acids were serine and threonine, the isoelectrical

point range was 9.0–6.5, limited proteolytic digestion with Staphylococcus aureus protease V8 yielded peptides of 43, 33 and 20 kDa and a polyclonal antibody raised against bovine brain pp50 cross-reacted on Western blot with the 52 kDa protein in SPM (Schrama, Pauloin, Louvard and Jollès, unpublished results). Taken together these data provide evidence that the 52 kDa protein is identical to the pp50 protein from coated vesicles.

The nervous tissue-specific phosphoprotein B-50 and LTP

Routtenberg's group (Routtenberg, 1985; Lovinger et al., 1986) reported an increase in F1 phosphorylation in a post-hoc *in vitro* phosphorylation assay after tetanization *in vivo*. We have shown that *in vitro* tetanization of hippocampal slices results in an increase in post-hoc phosphorylation of B-50, which is correlated with the degree of potentiation of the PS amplitude (Schrama et al., 1986a).

We have set up a new detection system to monitor the *in situ* phosphorylation of B-50 in order to establish the changes in its degree of phosphorylation under physiological conditions unambiguously. By incubation of hippocampal slices in Krebs buffer containing $^{32}P_i$ the endogenous ATP pool was labelled with ^{32}P. Termination of all phosphorylation and dephosphorylation reactions allowed accurate detection of B-50 phosphorylation by quantitative immunoprecipitation (De Graan et al., 1988a).

Depolarization of hippocampal slices with 30 mM K^+, a condition known to evoke NT release, stimulated B-50 phosphorylation. The depolarization-induced B-50 phosphorylation appeared to be due to enhanced PKC activity, because it could be blocked by the PKC inhibitor polymyxin B. Depolarization of slices in the absence of extracellular Ca^{2+} failed to stimulate B-50 phosphorylation and NT release (Dekker et al., 1988). Thus, PKC activation and subsequent B-50 phosphorylation appeared to be correlated with short-term effects on NT release.

Phorbol esters, potent activators of PKC, have been reported to enhance NT release (Versteeg and Ulenkate, 1987; Kaczmarek, 1987) and to induce LTP-like effects in hippocampal slices (Malenka et al., 1986) or to enhance tetanus-induced LTP *in vivo* (Routtenberg et al., 1986). Significant stimulation of B-50 phosphorylation in slices by 4β-phorbol 12,13-dibutyrate (PDB) was already detectable after 5 min and reached its maximum of about 300% after 30 min as compared to controls. The stimulation by 4β-phorbol 12-myristate 13-acetate (PMA) was much slower and reached significance after 30 min. After 30 min treatment, significant stimulation with PDB and PMA is found at 10^{-8} and 10^{-7} M, resp. The inactive 4α-phorbol 12,13-didecanoate (4α-PDD) did not affect B-50 phosphorylation (Table 1). Treatment with the membrane permeable synthetic diacylglycerol derivative DOG for 30 min, stimulated B-50 phosphorylation in a concentration dependent manner (De Graan et al., 1988b). These results confirmed the earlier conclusion that the phosphorylation of B-50 is correlated with short-term effects on NT release. In order to determine the changes in B-50 in relation to long-term changes in plasticity, we investigated the proposed effect of phorbol esters to induce LTP both with respect to changes in physical properties of the membrane and with respect to *in situ* B-50 phosphorylation.

A 20 min treatment of hippocampal slices with 10^{-6} M PDB produced long-lasting effects in the responsiveness of the Schaffer collateral/CA1 system and in the degree of B-50 phosphorylation. A significant increase in the PS amplitude and EPSP slope and B-50 phosphorylation was found within 20 min. The 4α-PDD, inactive with respect to PKC stimulation, did not enhance PS amplitude nor the slope of the EPSP and has no effect on B-50 phosphorylation (Table 1, Fig. 1). Despite removal of PDB after 20 min by exten-

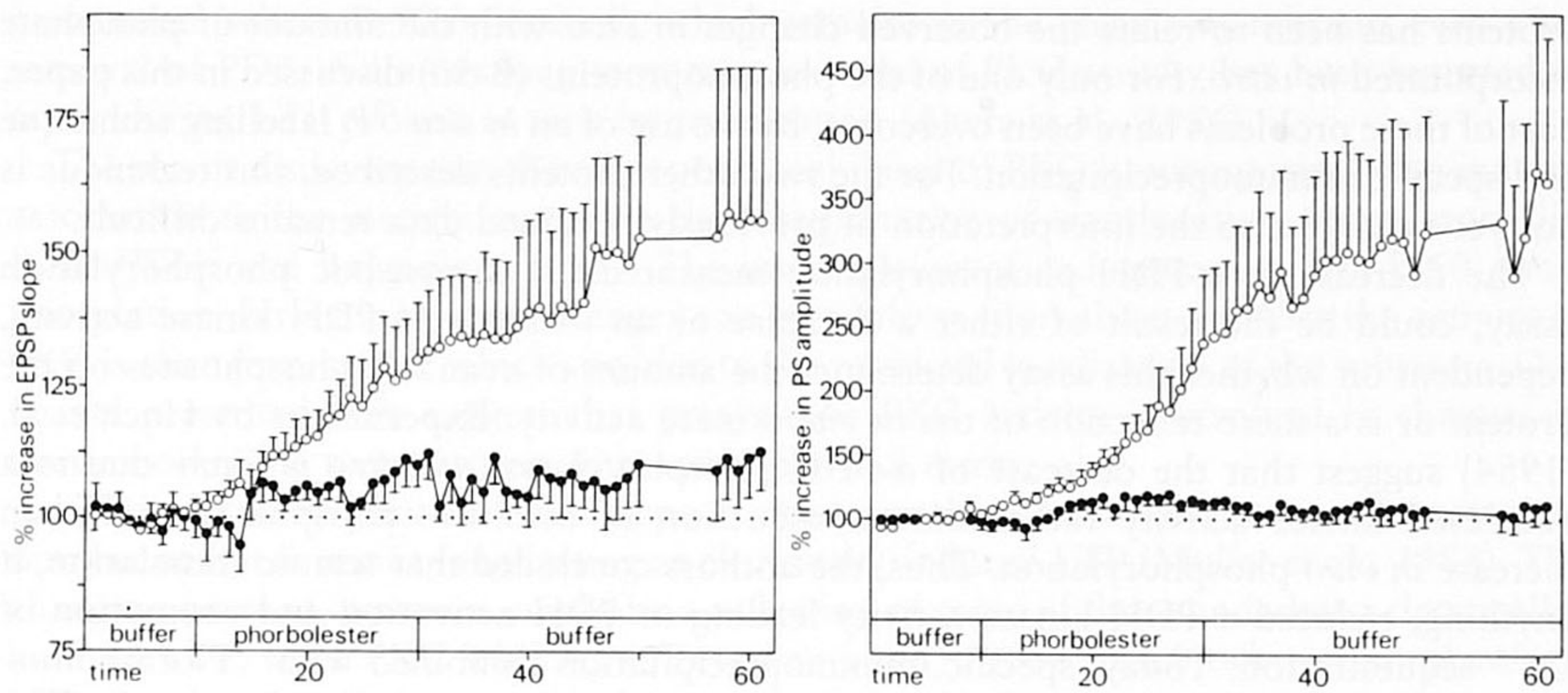

Fig. 1: Long-lasting effects of a 20 min phorbol treatment rising slope of the EPSP and on population spike (PS) amplitude. After treatment with 4α-PDD (closed circles) or 10^{-6} M PDB (open circles), slices were perfused with control Krebs buffer (see De Graan et al., 1988b). Field potentials were recorded in the stratum pyramidale of CA1 area of hippocampal slices. The average EPSP slope or PS amplitude is expressed as percentage (mean ± SEM) of the EPSP slope or PS amplitude, respectively, during the 10 min control period prior to the phorbol treatment.

Table 1: B-50 phosphorylation as percentage of control without phorbol at the same time point.

	30'	60'	90'
4α-PDD	91.7 ± 9.4	104.8 ± 8.5	96.8 ± 8.1
PDB	304.2 ± 12.1	309.4 ± 14.2	321.8 ± 18.5

Concentration used 1 μM. n = 4.

sive washing, B-50 phosphorylation remained elevated, 300% compared to the 4α-PDD controls for at least 60 min after removal of PDB (Table 1). Experiments in which tracer amounts of [^{3}H]-PDB were added, showed that 30 min after the removal of PDB less than 1% of the radioactivity remained in the slice. The PS amplitude continued to rise at least 30 min after removal of PDB, reaching 300% compared to control at 60 min (Fig. 1). The slope of the EPSP continued to rise for more than 30 min reaching its maximum of 150% at 60 min (Fig. 1). It could be concluded from these experiments, that the phosphorylation of B-50 is not only confined to short-term changes in plasticity, but also to longer-term changes as induced by phorbol esters.

Discussion

The correlation of LTP in mammals with membrane phosphorylation processes has revealed that the phosphorylation of at least 3 proteins is related to the changes observed in plasticity. All 3 proteins have been characterized and attempts have been made to identify the kinases involved. A major problem in establishing the role of these phospho-

proteins has been to relate the observed changes *in vivo* with the amount of phosphate incorporated *in vitro*. For only one of the phosphoproteins (B-50) discussed in this paper, part of these problems have been overcome, by the use of an *in situ* $^{32}P_i$ labelling technique and specific immunoprecipitation. For the two other proteins described, this technique is not yet available, so the interpretation of previously obtained data remains difficult.

The decrease in α-PDH phosphorylation measured, by a post-hoc phosphorylation assay, could be the result of either a decrease or an increase of PDH kinase activity, dependent on whether this assay determines the amount of available phosphosites on the protein or is a mere reflection of the *in vivo* kinase activity. Experiments by Hoch et al. (1984) suggest that the decrease of α-PDH phosphorylation *in vitro* is likely due to a decreased kinase activity rather than a reduction of available phosphosites after an increase *in vivo* phosphorylation. Thus, the authors concluded that tetanic stimulation, if anything, reduced α-PDH kinase activity leading to PDH activation and promotion of Ca^{2+} sequestration. Today, specific immunoprecipitation combined with ^{32}P-orthophosphate labelling could be of help to clarify the direction of change of phosphorylation of α-PDH during initiation of LTP. This could shed more light on the attractive hypothesis, by Browning et al. (1982), that there is a temporal increase in intracellular Ca^{2+} due to a decreased sequestration as a result of inactivation of PDH by phosphorylation shortly after application of a tetanus.

The role of the increase of pp50 phosphorylation as determined after the application of a tetanus is still poorly understood. It has been suggested that an increase in phosphorylation, destabilizes the interaction between certain coat proteins and the vesicle, thus leading to decoating of the vesicle (see Schrama et al., 1986a). Keen and Black (1986) have studied the *in situ* phosphorylation of CV proteins with $^{32}P_i$ in dissociated rat sympathetic neurons. In order to determine the *in situ* phosphorylation of pp50, they used a CV co-assembly assay, in which phosphorylated components from the labelled disrupted sympathetic neurons are allowed to assemble to intact CVs with purified clathrin from bovine brain. Then, the incorporated, phosphorylated proteins were detected after purification of intact CVs by gradient centrifugation. If phosphorylation of pp50 is associated with decoating of the vesicles, than this would be a unsatisfactory method to determine the *in situ* phosphorylation of pp50. The polyclonal antibodies raised to pp50 by the group of Pauloin (Paris, F) could be of great help to determine whether pp50 is phosphorylated *in vivo*. It is also still not known how the phosphorylation of pp50 is regulated, since there are no modulators of its kinase known. A recent report from Pauloin et al., (1988) indicates that the degree of phosphorylation of pp50 is regulated by the ATP/ADP ratio.

The third phosphoprotein described in this paper is by far the best characterized of the three discussed and of which the kinase (PKC) is thoroughly studied. Recent studies using molecular biological techniques have disclosed various subtypes of PKC including Ca^{2+}-independent types (Nishizuka, 1988). The substrate-specificity of these subtypes has not yet been unraveled and all the studies investigating the role of PKC and its substrates in LTP have made no distinction between the various types. Whatever the type of PKC phosphorylating B-50 may be, it seems that the phosphorylation of this protein is changed after the induction of LTP. From our studies in hippocampal slices with *in situ* phosphorylation it seems clear that the induction of LTP (with PDB and PMA) is correlated with an increase in B-50 phosphorylation, which is due to PKC activation. The B-50 phosphorylation remained elevated after removal of PDB, suggesting a sustained increase in PKC activity due to PDB treatment.

Phorbol esters are known to induce translocation of PKC (Kraft and Anderson, 1983). Therefore the sustained B-50 phosphorylation observed after removal of PDB from the slices, can be explained by a translocation or thighter binding of PKC to the presynaptic

membrane (where B-50 is located), which remains at the plasma membrane, even after removal of PDB. A similar long-term translocation of PKC activity has been reported to occur during LTP evoked *in vivo* by tetanization (Akers et al., 1986).

The recent discovery that the most potent inhibitor of PKC (staurosporine) also induces association to the membrane makes the interpretation of translocation assays more difficult (Wolf and Baggiolini, 1988). The investigation of endogenous *in situ* B-50 phosphorylation during long-term changes can give information about whether the activity of PKC is changing, and at which site, due to the restricted localization of the substrate. Our results presented here suggest that presynaptic PKC activity is involved in changes in transmission due to treatment with active phorbol esters.

LTP induced by phorbol esters appears not to be the same as that induced by tetanization because it is not as long lasting as electrically-induced LTP (Muller et al., 1988). The changes observed, however, might be a reflection of some of those underlying electrically-induced LTP, since PKC inhibitors (Reymann et al., 1988a) can block certain phases of LTP. Recent experiments suggest that not the induction but an aspect of the maintenance of LTP is mediated by PKC (Muller et al., 1988; Reymann et al., 1988b; Lovinger et al., 1987; Gustafsson et al., 1988).

Induction of LTP can be evoked by a postsynaptic Ca^{2+} influx as was demonstrated very recently by photolysis of nitr-5 (a photolabel Ca^{2+}-chelator) (Malenka et al., 1988). In a physiological situation, this Ca^{2+} influx is thought to result from high frequency stimulation of the hippocampus involving activation of NMDA receptor (Collingridge and Bliss, 1987; Linden et al., 1988). This receptor is coupled to a Ca^{2+} channel which is normally voltage-dependently blocked by Mg^{2+}. High frequency stimulation removes the Mg^{2+} from the channel, thereby allowing Ca^{2+} influx through this receptor-linked channel.

Ca^{2+} plays an essential role in mechanisms underlying LTP, both pre- and postsynaptically. An increased Ca^{2+} content in tetanized slices has been reported (Kuhnt et al., 1985) as well as increased depolarization dependent Ca^{2+} entry (Agoston et al., 1986; Malenka et al., 1988). LTP can not be evoked in cells injected with EGTA intracellularly (Lynch et al., 1983) and induction of LTP in the Schaffer collaterals and in the perforant path is prohibited by antagonists of the NMDA receptor (Collingridge and Bliss, 1987). The amount of extracellular Ca^{2+} as measured with a Ca^{2+}-sensitive electrode is quantitatively related to the amount of potentiation (Melchers et al., 1988). Moreover, LTP can be evoked in the CA1 region by incubating hippocampal slices temporarily in a buffer containing high Ca^{2+} (Melchers et al., 1987). Although these data provide evidence for a postsynaptic mechanism in the induction of LTP, participation of presynaptic NMDA receptors can not be excluded (Errington et al., 1987). After the induction of LTP a depolarization-stimulated, Ca^{2+}-dependent glutamate release is observed (Bliss and Lynch, 1988). In addition, changes in synaptic vesicle distribution 20 min after tetanization of the Schaffer collaterals have been reported (Applegate et al., 1987) also suggesting that changes in release mechanisms are involved in LTP. Not only Ca^{2+}, but also the Ca^{2+}-binding protein calmodulin (CaM) has been implicated in LTP (Lynch and Bliss, 1986; Reymann et al., 1988b). CaM is well known to participate in many ways in transmitter release mechanisms (Augustine et al., 1987).

Taking together, LTP seems to be accompanied by a sustained release of NT (e.g. glutamate) and in certain areas in the hippocampus an activation of the NMDA receptor. How this presynaptic event is related to that observed at the postsynaptic membrane is still not clear. One possibility is a feedback signal in the form of arachidonic acid or one of its metabolites from the postsynaptic induction side to the presynaptic maintenance side (Williams and Bliss, 1988). Another possibility is that independent of postsynaptic NMDA

receptor activation, there is a direct action of high frequency stimulation of the afferent fibers on the presynaptic nerve terminal. Recent experiments indicate that postsynaptic activation of NMDA receptors is not sufficient in itself for eliciting LTP, but that stimulation of the afferent fibers is also necessary (Kauer et al., 1988).

The sustained increase in B-50 phosphorylation observed during LTP suggests an activation of presynaptic PKC, through an, at present, unknown mechanism. While it is to be expected that PKC exerts its role through phosphorylation of its substrates, it is still unknown what the physiological role of (phosphorylation of) its substrates are. The recent discovery that B-50 is identical to the CaM-binding protein P-57 (Wakim et al., 1987), may shed new light on the possible function of this protein. Andreasen et al. (1983) have suggested that P-57 may act as a CaM buffer within the neuron, binding CaM at resting Ca^{2+} levels in the cell, but releasing it at elevated Ca^{2+} levels, as was determined *in vitro*. Phosphorylation of P-57 by PKC seems to impair CaM reassociation with the protein at low Ca^{2+} levels (Alexander et al., 1987). This would provide an attractive mechanism for long-term enhancement in synaptic efficacy, as a long-term increase in the free CaM will render the neuron more sensitive to stimulation.

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Summary

Protein phosphorylation is one of the major regulatory processes, involved in long-term changes in synaptic efficacy. The phosphorylation of three synaptic proteins, of 43 kDa (α-subunit of pyruvate dehydrogenase (PDH), 52 kDa (pp50, coated vesicle/coated pit phosphoprotein) and 48 kDa (B-50/F1/GAP-43) have been described. The activity of PDH is regulated by phosphorylation of the α-subunit, thereby regulating Ca^{2+} sequestration in the neuron. Phoshorylation of the 52 kDa protein might be involved in plasmamembrane recycling, after the increased neurotransmitter release seen in LTP. The phosphorylation of the neuron-specific presynaptic PKC substrate B-50 is proposed to be correlated with enhanced transmitter release during long-term potentiation of synaptic efficacy.

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